**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 48/00, C07H 21/04
C12N 5/10, 15/70

A1

(11) International Publication Number: WO 94/07538

(43) International Publication Date: 14 April 1994 (14.04.94)

(21) International Application Number: PCT/US93/09013

(22) International Filing Date: 23 September 1993 (23.09.93)

(30) Priority data:
956,698 1 October 1992 (01.10.92) US

(60) Parent Application or Grant
(63) Related by Continuation
US 956,698 (CIP)
Filed on 1 October 1992 (01.10.92)

(71) Applicant *(for all designated States except US)*: THOMAS JEFFERSON UNIVERSITY [US/US]; 1020 Locust Street, Philadelphia, PA 19107-6799 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : CALABRETTA, Bruno [IT/US]; 2401 Pine Street, Philadelphia, PA 19103 (US).

(74) Agent: MONACO, Daniel, A.; Seidel, Gonda, Lavorgna & Monaco, Two Penn Center Plaza, Suite 1800, Philadelphia, PA 19102 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
*With international search report.*

(54) Title: ANTISENSE OLIGONUCLEOTIDES TO B-myb PROTO-ONCOGENE

(57) Abstract

Oligonucleotides are provided having a nucleotide sequence complementary to at least a portion of the mRNA transcript of the B-myb gene. These "antisense" oligonucleotides are hybridizable to the B-myb mRNA transcript. Such oligonucleotides are useful in treating neoplastic diseases characterized by activation of B-myb gene expression.

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| AT | Austria | FR | France | MR | Mauritania |
| AU | Australia | GA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NE | Niger |
| BE | Belgium | GN | Guinea | NL | Netherlands |
| BF | Burkina Faso | GR | Greece | NO | Norway |
| BG | Bulgaria | HU | Hungary | NZ | New Zealand |
| BJ | Benin | IE | Ireland | PL | Poland |
| BR | Brazil | IT | Italy | PT | Portugal |
| BY | Belarus | JP | Japan | RO | Romania |
| CA | Canada | KP | Democratic People's Republic of Korea | RU | Russian Federation |
| CF | Central African Republic | | | SD | Sudan |
| CG | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SI | Slovenia |
| CI | Côte d'Ivoire | LI | Liechtenstein | SK | Slovak Republic |
| CM | Cameroon | LK | Sri Lanka | SN | Senegal |
| CN | China | LU | Luxembourg | TD | Chad |
| CS | Czechoslovakia | LV | Latvia | TG | Togo |
| CZ | Czech Republic | MC | Monaco | UA | Ukraine |
| DE | Germany | MG | Madagascar | US | United States of America |
| DK | Denmark | ML | Mali | UZ | Uzbekistan |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

# ANTISENSE OLIGONUCLEOTIDES TO B-myb PROTO-ONCOGENE

## Field of the Invention

The invention relates to antisense oligonucleotides to proto-oncogenes, in particular antisense oligonucleotides to the B-myb gene, and the use of such oligonucleotides as antineoplastic agents.

## Reference to Government Grant

The invention described herein was made in part with government support under grant CA46782 awarded by National Institutes of Health. The government has certain rights in the invention.

## Background of the Invention

Two genes, A-myb and B-myb, have recently been isolated that are related to the proto-oncogene c-myb, the cellular homolog of the transforming gene of the avian myeloblastosis virus (AMV) and the avian leukemia virus E26 which causes myeloblastic leukemia in chickens and transform myelomonocytic hematopoietic cells in culture.

Recent evidence suggests that c-myb plays an important role in the regulation of normal and leukemic hematopoiesis and T-lymphocyte proliferation. See, for example, Gewirtz and Calabretta, Science 242, 1303 (1988). Inhibition of c-myb expression prevents $G_1$/S transition in normal T-lymphocytes and is associated with selective down-regulation of DNA p lym rase-α expression (Venturelli et al., Proc. Natl. Acad. Sci. USA 87, 5963

(1989)), suggesting direct involvement of c-myb in an essential biochemical pathway leading to DNA synthesis. Moreover, constitutive expression of the human c-myb gene in murine fibroblasts abrogates the requirement for the cell cycle progression factor IGF-1 (Travali et al., Mol. Cell. Biol. 11, 731 (1990), further supporting the notion that c-myb plays a general role in cellular proliferation. The inhibition of normal and leukemic cells with antisense oligonucleotides which hybridize to the c-myb mRNA is described in U.S. Patent 5,098,890, the entire disclosure of which is incorporated herein by reference. The c-myb gene is necessary for proliferation of both normal and leukemic hematopoietic cells.

The product of the c-myb gene is a nuclear binding protein. B-myb is homologous to c-myb in the DNA binding domain and its pattern of expression does not appear to be restricted to hematopoietic cells. The cDNA nucleotide sequence of the human B-myb gene, and the deduced 700-amino acid sequence, are set forth in Nomura et al., Nucleic Acids Res. 16, 11705-11089 (1988), the entire disclosure of which is incorporated herein by reference. Like c-myb, B-myb acts as a transactivating factor. In transient expression assays, a constitutively expressed B-myb cDNA transactivates a reporter gene linked to the SV40 early promoter and enhancer through interaction of the encoded protein with B-myb binding sites in the SV40 early promoter (Mizuguchi et al., J. Biol. Chem. 265, 9280 (1990)).

The expression of the B-myb in normal hematopoietic cells has been shown to correlate with proliferation and c-myb expression in these cells (Golay et al., Blood 77, 149 (1991)). B-myb is also expressed in Balb/c3T3 fibroblasts at the $G_1$/S boundary, and appears to be growth-regulated at that boundary. H wever, despite the recent advances in the study of the B-myb

g ne, its role in maintaining proliferation of transformed cells has not been demonstrated.

## Summary of the Invention

The invention provides antisense oligonucleotides and pharmaceutical compositions thereof with pharmaceutically acceptable carriers. Each oligonucleotide has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the human B-myb gene. The oligonucleotide is hybridizable to the mRNA transcript, but does not hybridize to the c-myb gene. The oligonucleotide is at least an 8-mer oligonucleotide, that is, an oligomer containing at least 8 - nucleotide residues, and contains up to 50 nucleotides. In particular, the oligomer is advantageously a 12-mer to a 40-mer, preferably an oligodeoxynucleotide. While oligonucleotides smaller than 12-mers may be utilized, they are statistically more likely to hybridize with non-targeted sequences, and for this reason may be less specific. In addition, a single mismatch may destabilize the hybrid. While oligonucleotides larger than 40-mers may be utilized, uptake may be more difficult. Moreover, partial matching of long sequences may lead to non-specific hybridization, and non-specific effects. Most preferably, the oligonucleotide is a 15- to 30-mer oligodeoxynucleotide, more advantageously an 18- to 26-mer.

While in principle oligonucleotides having a sequence complementary to any region of the B-myb mRNA find utility in the present invention, oligonucleotides complementary to a portion of the B-myb mRNA transcript including the translation initiation codon are particularly preferred. Also preferred are oligonucleotides complementary to a portion of the B-myb mRNA transcript lying within ab ut 50 nucleotides (preferably within

about 40 nucleotides) upstream (the 5' direction), or about 50 (preferably 40) nucleotides downstream (the 3' direction) from the translation initiation codon.

The invention provides a method of treating neoplastic disease in vivo or ex vivo comprising administering to an individual or cells harvested from the individual an effective amount of B-myb antisense oligonucleotide. The neoplastic diseases treatable include those diseases in which the B-myb gene is activated.

The invention is also a method for purging bone marrow of neoplastic cells. Bone marrow aspirated from an inflicted individual is treated with an effective amount of B-myb antisense oligonucleotide, and the thus-treated cells are then returned to the body of the afflicted individual.

According to another embodiment, the invention relates to an artificially-constructed gene comprising a transcriptional promotor segment and a segment containing B-myb DNA in inverted orientation such that transcription of the artificially-constructed gene produces RNA complementary to at least a portion of the mRNA transcript of the B-myb gene. The gene may be introduced into cells which are characterized by the activation of B-myb expression to inhibit the proliferation of those cells. The artificially-constructed gene may be introduced into the neoplastic cells by, for example, transfection, transduction with a viral vector, or microinjection.

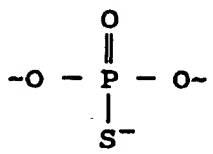
As used in the herein specification and appended claims, unless otherwise indicated, the term "oligonucleotide" includes both oligomers of ribonucleotides, i.e., oligoribonucleotides, and oligomers of deoxyribonucleotides, i.e., oligodeoxyribonucleotides

(also referred to herein as "oligodeoxynucl otides"). Oligodeoxynucleotides are preferred.

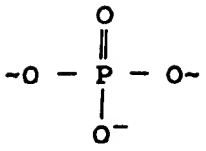
As used herein, unless otherwise indicated, the term "oligonucleotide" also includes oligomers which may be large enough to be termed "polynucleotides".

The terms "oligonucleotide" and "oligodeoxynucleotide" include not only oligomers and polymers of the common biologically significant nucleotides, i.e., the nucleotides adenine ("A"), deoxyadenine ("dA"), guanine ("G"), deoxyguanine ("dG"), cytosine ("C"), deoxycytosine ("dC"), thymine ("T") and uracil ("U"), but also include oligomers and polymers hybridizable to the B-myb mRNA transcript which may contain other nucleotides. Likewise, the terms "oligonucleotide" and "oligodeoxynucleotide" includes oligomers and polymers wherein one or more purine or pyrimidine moieties, sugar moieties or internucleotide linkages is chemically modified. The term "oligonucleotide" is thus understood to also include oligomers which may properly be designated as "oligonucleosides" because of modification of the internucleotide phosphodiester bond. Such modified oligonucleotides include, for example, the alkylphosphonate oligonucleosides, discussed below.

The term "phosphorothioate oligonucleotide" means an oligonucleotide wherein one or more of the internucleotide linkages is a phosphorothioate group,

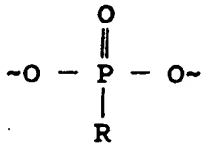


as opposed to the phosphodiester group



which is characteristic of unmodified oligonucleotides.

By "alkylphosphonate oligonucleoside" is meant an oligonucleotide wherein one or more of the internucleotide linkages is an alkylphosphonate group,



wherein R is an alkyl group, preferably methyl or ethyl.

The term "downstream" when used in reference to a direction along a nucleotide sequence means the 5′→3′ direction. Similarly, the term "upstream" means the 3′→5′ direction.

The term "B-myb mRNA transcript" means the presently known mRNA transcript of the human B-myb gene and all variations thereof, or any further transcripts which may be elucidated.

## Description of the Figures

Fig. 1A shows a Balb/c3T3 cell culture transfected with pSV40 polylinker (control).

Fig. 1B shows a Balb/c3T3 cell culture transfected with a pSV/B-myb construct.

Fig. 1C shows a Balb/c3T3 cell culture transfected with a pSV/anti-B-myb construct (antisense orientation).

Fig. 2A is similar to Fig. 1B and shows a LAN-5 neuroblastoma cell culture transfected with the pSV/B-myb construct.

Fig. 2B is similar to Fig. 1C and shows a LAN-5 cell culture transfected with the pSV/anti-B-myb construct.

Fig. 3 is a cell count of LAN-5 cells treated with B-myb antisense ("AS") or sense ("S") oligomer, or of untreated LAN-5 cells ("C").

## Detailed Description of the Invention

The putative DNA sequence complementary to the mRNA transcript of the human B-myb gene has been reported by Nomura et al., Nucleic Acids Res. 16, 11705-11089 (1988), the entire disclosure of which is incorporated herein by reference. These investigators further disclose the predicted 700 amino acid sequence of the putative B-myb protein. The initiation codon ATG is preceded by a 5'-untranslated region of about 127 nucleotides. The termination codon TGA is followed by a 3'-untranslated region, which includes a putative polyadenylation signal (AATAAA) located seventy nucleotides downstream of the termination codon.

The antisense oligonucleotides of the invention, which are complementary to the B-myb mRNA, may be synthesized by any of the known chemical oligonucleotide synthesis methods. Such methods are generally described, for example, in Winnacker, From Genes to Clones: Introduction to Gene Technology, VCH Verlagsgesellschaft mbH (Ibelgaufts trans. 1987). The antisense oligonucleotides are most advantageously prepared by utilizing any of the commercially available, automated nucleic acid synthesizers. One such device, the Applied Bi systems 380B DNA Synth sizer, utilizes $\beta$-cyan ethyl phosphoramidite chemistry.

Since the complete nucleotide synthesis of DNA complementary to the B-myb mRNA transcript is known, antisense oligonucleotides hybridizable with any portion of the mRNA transcript may be prepared by oligonucleotide synthesis methods known to those skilled in the art.

The B-myb and c-myb cDNA nucleotide sequences are characterized by varying degrees of homology in three distinct domains, corresponding to the following amino acid residues (I) B-myb 33-193, c-myb 42-202; (II) B-myb 468-545, c-myb 414-498; and (III) B-myb 663-681, c-myb 608-626. The homology is primarily in the DNA-binding domain. The strongest homology lies from about B-myb amino acid 63 to about 193.

Preferably, the antisense oligonucleotides of the invention are selected so as to be specific for hybridizing to B-myb, and not c-myb mRNA. The use of such B-myb specific antisense oligonucleotide would minimize the side effects which could result from inhibition of c-myb expression in normal cells. The combined effect of B-myb and c-myb expression inhibition might have an adverse effect particularly on hematopoietic cells, both of which are characterized by both c-myb and B-myb expression. The combination effect is avoided by selecting the portion of the B-myb mRNA targeted for hybridization from the regions which have a lesser degree of homology with the corresponding c-myb sequence. The selection of the particular sequence for the antisense oligonucleotide is therefore of necessity made not only on the basis of the B-myb mRNA nucleotide sequence, but also the c-myb sequence. The complete cDNA nucleotide sequence of the human c-myb gene is disclosed by Majello et al., Proc. Natl. Acad. Sci. U.S.A. 38, 9636-9640 (1986), and U.S. Patent 5,098,890,

the entire disclosures of both of which are incorporated herein by reference.

The areas of the B-myb mRNA which are most preferred for targeting for antisense hybridization are those sequences lying completely outside the regions of homology with c-myb, that is, the preferred areas for targeting include nucleotides 1-226, 708-1531, 1764-2116 and 2172-2630 (nucleotide 128 being the translation initiation codon).

While any length oligonucleotide may be utilized in the practice of the invention, sequences shorter than 12 nucleotides, and in particular sequences shorter than 8 nucleotides, may be less specific in hybridizing to the target mRNA, may be more easily destroyed by enzymatic digestion, and may be destabilized by enzymatic digestion. Hence, oligonucleotides having 12 or more nucleotides are preferred.

Long sequences, particularly sequences longer than about 50 nucleotides, may be somewhat less effective in inhibiting B-myb translation because of decreased uptake by the target cell. Thus, oligomers of 12-40 nucleotides are preferred, more preferably 15-30 nucleotides, most preferably 18-26 nucleotides. While sequences of 18-21 nucleotides are most particularly preferred for unmodified oligonucleotides, slightly longer chains of up to about 26 nucleotides, are preferred for modified oligonucleotides such as phosphorothioate oligonucleotides, which hybridize less strongly to mRNA than unmodified oligonucleotides. It may be appreciated that oligonucleotides appreciably larger than 50 nucleotides may be freely utilized where oligonucleotide delivery is via transfection of targeted cells with a vector coding for B-myb antisense oligonucleotide.

Oligonucleotides complementary to and hybridizable with any portion of the B-myb mRNA transcript are, in principle, effective for inhibiting translation of the transcript, and capable of inducing the effects herein described. It is believed that translation is most effectively inhibited by blocking the mRNA at a site at or near the initiation codon. This region shares essentially no homology with c-myb. Thus, oligonucleotides complementary to the 5'-terminal region of the B-myb mRNA transcript are preferred. The antisense oligonucleotide is preferably directed to a site at or near the initiation codon for protein synthesis. Oligonucleotides complementary to the B-myb mRNA, including the initiation codon (the first codon at the 5' end of the translated portion of the B-myb transcript) are preferred.

While antisense oligomers complementary to the 5'-terminal region of the B-myb transcript are preferred, particularly the region including the initiation codon, it should be appreciated that useful antisense oligomers are not limited to those complementary to the sequences found in the translated portion of the mRNA transcript, but also includes oligomers complementary to nucleotide sequences contained in, or extending into, the 5'-and 3'-untranslated regions.

The following 50-mer oligodeoxynucleotide is complementary to the B-myb mRNA transcript beginning with the initiation codon of the transcript and extending downstream thereof (in the 3' direction): SEQ ID NO:1.

Particularly preferred smaller oligonucleotides hybridizable to segments of the B-myb message containing the initiation codon are the oligomers having a nucleotid sequence selected from the nucleotide sequence of the foll wing 15- to 30-mers:

SEQ ID NO: 2,
SEQ ID NO: 3,
SEQ ID NO: 4,
SEQ ID NO: 5,
SEQ ID NO: 6,
SEQ ID NO: 7,
SEQ ID NO: 8,
SEQ ID NO: 9,
SEQ ID NO: 10,
SEQ ID NO: 11,
SEQ ID NO: 12,
SEQ ID NO: 13,
SEQ ID NO: 14,
SEQ ID NO: 15,
SEQ ID NO: 16 and
SEQ ID NO: 17.

Smaller oligomers based upon the above sequence, in particular, oligomers hybridizable to segments of the B-myb message containing the initiation codon, may be utilized. Particularly preferred are oligomers containing at least 12 nucleotides, having a nucleotide sequence corresponding to a portion of SEQ ID NO:1.

The oligonucleotide employed may represent an unmodified or modified oligonucleotide. Thus, oligonucleotides hybridizable to the B-myb mRNA transcript finding utility according to the present invention include not only oligomers of the biologically significant native nucleotides, i.e., A, dA, G, dG, C, dC, T and U, but also oligonucleotide species which have been modified for improved stability and/or lipid solubility. For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or alkoxy group for a phosphat oxygen in the internucleotide phosphodiester linkage to

form an alkylphosphonate oligonucleoside or alkylphosphotriester oligonucleotide. Non-ionic oligonucleotides such as these are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in U.S. Patent 4,469,863.

Methylphosphonate oligomers can be prepared by a variety of methods, both in solution and on insoluble polymer supports (Agrawal and Riftina, Nucl. Acids Res., 6, 3009-3024 (1979); Miller et al., Biochemistry, 18, 5134-5142 (1979), Miller et al., J. Biol. Chem., 255, 9659-9665 (1980); Miller et al., Nucl. Acids Res., 11, 5189-5204 (1983), Miller et al., Nucl. Acids Res., 11, 6225-6242 (1983), Miller et al., Biochemistry, 25, 5092-5097 (1986); Engels and Jager, Angew. Chem. Suppl. 912 (1982); Sinha et al., Tetrahedron Lett. 24, 877-880 (1983); Dorman et al, Tetrahedron, 40, 95-102 (1984); Jager and Engels, Tetrahedron Lett., 25, 1437-1440 (1984); Noble et al., Nucl. Acids Res., 12, 3387-3404 (1984); Callahan et al., Proc. Natl. Acad. Sci. USA, 83, 1617-1621 (1986); Koziolkiewicz et al., Chemica Scripta, 26, 251-260 (1986); Agrawal and Goodchild, Tetrahedron Lett., 38, 3539-3542 (1987); Lesnikowski et al., Tetrahedron Lett., 28, 5535-5538 (1987); Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

The most efficient procedure for preparation of methylphosphonate oligonucleosides involves use of 5'-O-dimethoxytrityldeoxynucleoside-3'-O-diisopropylmethylphosphoramidite intermediates, which are similar to th methoxy or $\beta$-cyan ethyl phosphoramidite r agents used to pr pare oligodeoxyribonucleotides. Th

methylphosphonate oligomers can be prepared on controlled pore glass polymer supports using an automated DNA synthesizer (Sarin et al., Proc. Natl. Acad. Sci. USA, 85, 7448-7451 (1988)).

Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoroamidites according to the procedure of Dagle et al., Nucl. Acids Res. 18, 4751-4757 (1990).

Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the property of cellular uptake via a receptor (Loke et al., Proc. Natl. Acad. Sci. U.S.A. 86, 3474-3478 (1989)).

Phosphorothioate oligodeoxynucleotide are described by LaPlanche, et al., Nucleic Acids Research 14, 9081 (1986) and by Stec et al., J. Am. Chem. Soc. 106, 6077 (1984). The general synthetic method for phosphorothioate oligonucleotides was modified by Stein et al., Nucl. Acids Res., 16, 3209-3221 (1988), so that these compounds may readily be synthesized on an automatic synthesizer using the phosphoramidite approach.

Furthermore, recent advances in the production of oligoribonucleotide analogues mean that other agents may also be used for the purposes described here, e.g., 2'-O-methylribonucleotides (Inove et al., Nucleic Acids Res. 15, 6131 (1987) and chimeric oligonucleotides that are composite RNA-DNA analogues (Inove et al., FEBS Lett. 215, 327 (1987).

While inhibition of B-myb mRNA translation is possible utilizing either antisense oligoribonucleotides

or oligodeoxyribonucleotides, free oligoribonucleotides are more susceptible to enzymatic attack by ribonucleases than oligodeoxyribonucleotides. Hence, oligodeoxyribonucleotides are preferred in the practice of the present invention. Oligodeoxyribonucleotides are further preferred because, upon hybridization with B-myb mRNA, the resulting DNA-RNA hybrid duplex is a substrate for RNase H, which specifically attacks the RNA portion of DNA-RNA hybrid. Degradation of the mRNA strand of the duplex releases the antisense oligodeoxynucleotide strand for hybridization with additional B-myb messages.

In general, the antisense oligonucleotides used in the method of the present invention will have a sequence which is completely complementary to the target portion of the B-myb message. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "nucleotide sequence complementary to at least a portion of the mRNA transcript" of B-myb does not necessarily mean a sequence having 100% complementarity with the transcript. In general, any oligonucleotide having sufficient complementarity to form a stable duplex with B-myb mRNA, that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target region of the B-myb message. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. More than one mismatch probably will not be tolerated for antisense oligomers of less than about 21 nucleotides. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any given antisense oligomer and the target B-myb message sequence, bas d upon the m lting point, and ther - fore the stability, of the r sulting duplex. M lting

points of duplexes of a given base pair composition can be readily determined from standard texts, such as Molecular Cloning: A Laboratory Manual, (2nd edition, 1989), J. Sambrook et al., eds.

While oligonucleotides capable of stable hybridization with any region of the B-myb message are within the scope of the present invention, oligonucleotides complementary to a region including the translation initiation codon are believed particularly effective. Particularly preferred are oligonucleotides hybridizable to a region of the B-myb mRNA up to 40 nucleotides upstream (in the 5' direction) of the initiation codon or up to 40 nucleotides downstream (in the 3' direction) of that codon.

For therapeutic use, the antisense oligonucleotides may be combined with a pharmaceutical carrier, such as a suitable liquid vehicle or excipient and an optional auxiliary additive or additives. The liquid vehicles and excipients are conventional and commercially available. Illustrative thereof are distilled water, physiological saline, aqueous solution of dextrose, and the like. The B-myb mRNA antisense oligonucleotides are preferably administered parenterally, most preferably intravenously. The vehicle is designed accordingly. Alternatively, oligonucleotide may be administered subcutaneously via controlled release dosage forms.

In addition to administration with conventional carriers, the antisense oligonucleotides may be administered by a variety of specialized oligonucleotide delivery techniques. For example, oligonucleotides may be encapsulated in liposomes for therapeutic delivery. The oligonucleotide, depending upon its solubility, may be present both in the aque us layer and in the lipidic layer, or in what is generally termed a lip s mic sus-

pension. The hydrophobic layer, generally but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, ionic surfactants such as diacetylphosphate, stearylamine, or phosphatidic acid, and/or other materials of a hydrophobic nature. Oligonucleotides have been successfully encapsulated in unilamellar liposomes.

Reconstituted Sendai virus envelopes have been successfully used to deliver RNA and DNA to cells. Arad et al., Biochem. Biophy. Acta. 859, 88-94 (1986).

The oligonucleotides may be conjugated to poly(L-lysine) to increase cell penetration. Such conjugates are described by Lemaitre et al., Proc. Natl. Acad. Sci. USA, 84, 648-652 (1987). The procedure requires that the 3'-terminal nucleotide be a ribonucleotide. The resulting aldehyde groups are then randomly coupled to the epsilon-amino groups of lysine residues of poly(L-lysine) by Schiff base formation, and then reduced with sodium cyanoborohydride. This procedure converts the 3'-terminal ribose ring into a morpholine structure antisense oligomers.

The oligonucleotides may be conjugated for therapeutic administration to ligand-binding molecules which recognize cell-surface molecules, such as according to International Patent Application WO 91/04753. In particular, transferrin-polylysine-oligonucleotide complexes may be prepared for uptake by cells expressing high levels of transferrin receptor. The preparation of such complexes as carriers of oligonucleotide uptake into cells is described by Wagner et al., Proc. Natl. Acad. Sci. USA 87, 3410-3414 (1990). Inhibition of leukemia cell proliferation by transferrin receptor-mediated uptake of c-myb antisense oligonucleotides conjugated to transferrin was demonstrated by Citro et al., Proc. Natl. Acad. Sci. USA 89, 7031-7035 (1992).

The disorders treatable with the antisense oligonucleotides of the invention include neoplastic diseases characterized by the activation of B-myb expression, signalled by the appearance of B-myb mRNA transcripts and/or the 700-amino acid protein product. The level of B-myb expression may be determined, for example, by probing total cellular RNA from tumor cells with a complementary probe for B-myb mRNA, according to Nomura et al., Nucleic Acids Res. 16 (28), 11075-11089 (1988). Total cytoplasmic RNA from the tumor cells is passed over oligo (dT)-cellulose. The glyoxylated poly(A)$^+$ is fractionated on a 0.7% agarose gel, transferred to a filter and hybridized to an appropriately labelled nucleic acid probe for B-myb mRNA. The number of B-myb mRNA transcripts found in the tumor cells is compared to that found in normal cells from the same tissue. An at least 10-fold increase in B-myb expression in neoplastic cells over expression in normal cells from the same tissue indicates activation of B-myb expression, and would indicate that the disease would respond to B-myb antisense treatment. This threshold is based upon correlations between the level of gene expression and the extent of the disease state for various other oncogenes. See, for example, Slamon et al., Science 235, 177-182 (1988) and Science, 244, 707-712 (1989) (correlation between erb-b2 amplification/expression and breast or ovarian cancer); Alitalo et al., Advances in Cancer Research, 47, 235-282 (1986).

Disease conditions characterized by B-myb activated expression include, for example, neuroectodermal cancers such as neuroblastoma and neuroepithelioma, malignant melanoma, breast cancer, prostate carcinoma, colon cancer, renal carcinoma and leukemia and lymphoma.

A preferred method of administration of oligonucl otid comprises ither r gional or systemic per-

fusion, as is appropriat . According to a method of regional perfusion, the afferent and efferent vessels supplying the extremity containing the lesion are isolated and connected to a low-flow perfusion pump in continuity with an oxygenator and a heat exchanger. The iliac vessels may be used for perfusion of the lower extremity. The axillary vessels are cannulated high in the axilla for upper extremity lesions. Oligonucleotide is added to the perfusion circuit, and the perfusion is continued for an appropriate time period, e.g., one hour. Perfusion rates of from 100 to 150 ml/minute may be employed for lower extremity lesions, while half that rate should be employed for upper extremity lesions. Systemic heparinization may be used throughout the perfusion, and reversed after the perfusion is complete. This isolation perfusion technique permits administration of higher doses of chemotherapeutic agent than would otherwise be tolerated upon infusion into the arterial or venous systemic circulation.

For systemic infusion, the oligonucleotides are preferably delivered via a central venous catheter, which is connected to an appropriate continuous infusion device. Indwelling catheters provide long term access to the intravenous circulation for frequent administration of drugs over extended time periods. They are generally surgically inserted into the external cephalic or internal jugular vein under general or local anesthesia. The subclavian vein is another common site of catheterization. The infuser pump may be external, or may form part of an entirely implantable central venous system such as the INFUSAPORT system available from Infusaid Corp., Norwood, MA and the PORT-A-CATH system available from Pharmacia Laboratories, Piscataway, NJ. Th se d vices are implanted into a subcutaneous pocket under local anesth sia. A catheter, connected to the

pump injection port, is threaded through the subclavian vein to the superior vena cava. The implant contains a supply of oligonucleotide in a reservoir which may be replenished as needed by injection of additional drug from a hypodermic needle through a self-sealing diaphragm in the reservoir. Completely implantable infusers are preferred, as they are generally well accepted by patients because of the convenience, ease of maintenance and cosmetic advantage of such devices.

The antisense oligonucleotides may also be administered locally, as contrasted to regional or systemic administration. Local administration of polynucleotides have been carried out by direct injection into muscle. Local administration of oligonucleotides may be particularly useful in treating neuroectodermal tumors and melanoma. A pharmaceutical preparation of antisense oligonucleotide may be delivered locally to the tumor site by means of a catheter. Such catheters have been used to deliver drugs for local cardiovascular treatment and can be adapted for use in delivering drug directly to neuroplastic lesions. For treatment of melanoma, the oligonucleotides may be delivered by skin infiltration. Methods for delivering therapeutic oligonucleotide and polynucleotides by local infiltration are known to those skilled in the art.

As an alternative to treatment with exogenous oligonucleotide, antisense oligonucleotide synthesis may be induced <u>in situ</u> by local treatment of the targeted neoplastic cell with a vector containing an artificially-constructed gene comprising a transcriptional promotor and B-<u>myb</u> DNA in inverted orientation. The B-<u>myb</u> for insertion into the artificial gene in inverted orientation comprises cDNA which may be prepared, for example, by reverse transcriptase polymerase chain reacti n fr m RNA using primers derived from the published cDNA

sequence of B-myb. Upon transcription, the inverted B-myb gene segment, which is complementary to at least a portion of the B-myb mRNA, is produced in situ in the targeted cell. The endogenously produced RNA hybridizes to B-myb mRNA, resulting in interference with B-myb function and inhibition of the proliferation of the targeted cell.

The promotor segment of the artificially-constructed gene serves as a signal conferring expression of the inverted B-myb sequence which lies downstream thereof. It will include all of the signals necessary for initiating transcription of the sequence. The promotor may be of any origin as long as it specifies a rate of transcription which will produce sufficient antisense mRNA to inhibit the expression of the B-myb gene, and therefore the proliferation of the tumor cells. Preferably, a highly efficient promotor such as a viral promotor is employed. Other sources of potent promotors include cellular genes that are expressed at high levels. The promotor segment may comprise a constitutive or a regulatable promotor. Described in the hereinafter Example 1 is a typical construct which utilizes the SV40 promotor.

The artificial gene may be introduced by any of the methods described in U.S. Patent 4,740,463, incorporated herein by reference. One technique is transfection, which can be done by several different methods. One method of transfection involves the addition of DEAE-dextran to increase the uptake of the naked DNA molecules by a recipient cell. See McCutchin, J.H. and Pagano, J.S., J. Natl. Cancer Inst. 41, 351-7 (1968). Another method of transfection is the calcium phosphate precipitation technique which depends upon the addition of $Ca^{++}$ to a phosphate-containing DNA solution. The resulting precipitate apparently includes DNA in

association with calcium phosphate crystals. These crystals settle onto a cell monolayer; the resulting apposition of crystals and cell surface appears to lead to uptake of the DNA. A small proportion of the DNA taken up becomes expressed in a transfectant, as well as in its clonal descendants. See Graham, F.L. and van der Eb, A.J., Virology 52, 456-467 (1973) and Virology 54, 536-539 (1973).

Transfection may also be carried out by cationic phospholipid-mediated delivery. In particular, polycationic liposomes can be formed from N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). See Felgner et al., Proc. Natl. Acad. Sci. USA 84, 7413-7417 (1987) (DNA-transfection); Malone et al., Proc. Natl. Acad. Sci. USA, 86, 6077-6081 (1989) (RNA-transfection).

Alternatively, the artificially-constructed gene can be introduced in to cells, in vitro or in vivo, via a transducing viral vector. See Tabin et al., Mol. Cel. Biol. 2, 426-436 (1982). Use of a retrovirus, for example, will infect a variety of cells and cause the artificial gene to be inserted into the genome of infected cells. Such infection could either be done with the aid of a helper retrovirus, which would allow the virus to spread through the organism, or the antisense retrovirus could be produced in a helper-free system, such as ψ2-like cells (See Mann et al., Cell 33, 153-160, 1983) that package amphotropic viruses. A helper-free virus might be employed to minimize spread throughout the organism. Viral vectors in addition to retroviruses can also be employed, such as paporaviruses, SV40-like viruses, or papilloma viruses. The use of retroviruses for gene transfer has been reviewed by Eglitis and Anderson, BioTechniques 6, 608-614 (1988).

Vesicle fusion could also be employed to deliver the artificial gene. Vesicle fusion may be physically targeted to the tumor tissue if the vesicle were approximately designed to be taken up by the cells containing B-myb. Such a delivery system would be expected to have a lower efficiency of integration and expression of the artificial gene delivered, but would have a higher specificity than a retroviral vector. A combination strategy of targeted vesicles containing papilloma virus or retrovirus DNA molecules might provide a method for increasing the efficiency of expression of targeted molecules.

Still another alternative is to introduce the artificial gene via micro-injection. See for example, Laski et al., Cell, 1982.

Particulate systems and polymers for in vitro and in vivo delivery of polynucleotides was extensively reviewed by Felgner in Advanced Drug Delivery Reviews 5, 163-187 (1990). Techniques for direct delivery of purified genes in vivo, without the use of retroviruses, has been reviewed by Felgner in Nature 349, 351-352 (1991). Such methods of direct delivery of polynucleotides may be utilized for local delivery of either exogenous B-myb antisense oligonucleotide or artificially-constructed genes producing B-myb antisense oligonucleotide in situ.

Recently, Wolf et al. demonstrated that direct injection of non-replicating gene sequences in a non-viral vehicle is possible. See Science, 247, 1465-1468 (1990). DNA injected directly into mouse muscle did not integrate into the host genome, and plasmid essentially identical to the starting material was recovered from the muscle months after injection. Interestingly, no special delivery system is required. Simpl saline or

sucrose solutions are sufficient to delivery DNA and RNA.

The B-myb antisense oligonucleotides may be administered to the patient in the form of an appropriate pharmaceutical composition. Alternatively, the antisense oligonucleotides may be administered ex vivo, to cells harvested from the patient. Thus, according to a preferred embodiment of the invention, the B-myb antisense oligonucleotides are utilized as bone marrow purging agents for in vitro cleansing of the patient's bone marrow contaminated by leukemic cells. The antisense oligonucleotides are believed useful as purging agents in either allogeneic or autologous bone marrow transplantation.

Many neoplasms, such as neuroblastoma, melanoma and breast cancer, may be substantially metastatic, particularly in advanced stages. In particular, malignant cells may metastasize to the bone marrow. Patients with disseminated disease may have bone marrow metastases. It is therefore necessary to develop an effective method to purge bone marrow of all remaining neoplastic cells if autologous bone marrow transplantation is used in conjunction with aggressive chemotherapy. According to the present invention, B-myb antisense oligonucleotides may be used as bone marrow purging agents for the in vitro cleansing of bone marrow of malignant cells which have metastasized to the bone marrow from other tissues.

According to a method for bone marrow purging, bone marrow is harvested from a donor by standard operating room procedures from the iliac bones of the donor. Methods of aspirating bone marrow from donors are well-known in the art. Examples of apparatus and processes for aspirating bone marrow from donors are disclosed in U.S. Patents 4,481,946 and 4,486,188, incorporated here-

in by reference. Sufficient marrow is withdrawn so that the recipient, who is either the donor (autologous transplant) or another individual (allogeneic transplant), may receive from about 4 x $10^8$ to about 8 x $10^8$ processed marrow cells per kg of bodyweight. This generally requires aspiration of about 750 to about 1000 ml of marrow. The aspirated marrow is filtered until a single cell suspension, known to those skilled in the art as a "buffy coat" preparation, is obtained. This suspension of leukocytes is treated with B-myb antisense oligonucleotides in a suitable carrier, advantageously in a concentration of about 50-100 $\mu$g/ml. Alternatively, the leucocyte suspension may be stored in liquid nitrogen using standard procedures known to those skilled in the art until purging is carried out. The purged marrow can be stored frozen in liquid nitrogen until ready for use. Methods of freezing bone marrow and biological substances are disclosed, for example, in U.S. Patents 4,107,937 and 4,117,881.

Other methods of preparing bone marrow for treatment with B-myb antisense may be utilized, which methods may result in even more purified preparations of hematopoietic cells than the aforesaid buffy coat preparation.

After treatment with the antisense oligonucleotides, the cells to be transferred are washed with autologous plasma or buffer to remove unincorporated oligomer. The washed cells are then infused back into the patient.

The amount of antisense oligonucleotide may vary depending on the nature and extent of the neoplasm, the particular oligonucleotide utilized, and other factors. The actual dosage administered may take into account the size and weight of the patient, whether the nature of th treatment is pr phylactic or therap utic

in nature, the age, health and sex of the patient, the route of administration, whether the treatment is regional or systemic, and other factors. Concentrations of from about 1 to about 100 μg/ml may be employed, preferably from about 10 μg/ml to about 100 μg/ml, most preferably from about 20 μg/ml to about 60 μg/ml. The patient should receive a sufficient daily dosage of antisense oligonucleotide to achieve these intercellular concentrations of drug. The daily dosage may range from about 0.1 to 1,000 mg oligonucleotide per day, preferably from about 10 to about 700 mg per day. Greater or lesser amounts of oligonucleotide may be administered, as required. Those skilled in the art should be readily able to derive appropriate dosages and schedules of administration to suit the specific circumstance and needs of the patient.

It is believed that a course of treatment may advantageously comprise infusion of the recommended daily dose of oligonucleotide for a period of from about 3 to about 28 days, more preferably from about 7 to about 10 days. Those skilled in the art should readily be able to determine the optimal dosage in each case. For modified oligonucleotides, such as phosphorothioate oligonucleotides, which have a half life of from 24 to 48 hours, the treatment regimen may comprise dosing on alternate days.

For an about 70 kg adult human being, a daily dose of about 350 mg oligonucleotide is believed sufficient, to achieve an effective extracellular concentration of 2-20 μM. For children, the daily dosage is reduced proportionately according to the weight of the patient.

For ex vivo antineoplastic application, such as, for example, in b ne marrow purging, the B-myb antisens oligonucleotides may be administered in

amounts effective to kill neoplastic cells. Such amounts may vary depending on the extent to which malignant cells may have metastasized to the bone marrow, the particular oligonucleotide utilized, the relative sensitivity of the neoplastic cells to the oligonucleotide, and other factors. Concentrations from about 10 to 200 $\mu$g/ml per $10^5$ cells may be employed, preferably from about 40 to 150 $\mu$g/ml per $10^5$ cells. Supplemental dosing of the same or lesser amounts of oligonucleotide are advantageous to optimize the treatment. Thus, for purging bone marrow containing 2 x $10^7$ cell per ml of marrow volume, dosages of from about 2 to 40 mg antisense per ml of marrow may be effectively utilized, preferably from about 8 to 24 mg/ml. Greater or lesser amounts of oligonucleotide may be employed.

The effectiveness of the treatment may be assessed by routine methods which are used for determining whether or not remission has occurred. Such methods generally depend upon some combination of morphological, cytochemical, cytogenetic, immunologic and molecular analyses. In addition, remission can be assessed genetically by probing the level of expression of the B-myb oncogene. The reverse transcriptase polymerase chain reaction methodology can be used to detect even very low numbers of mRNA transcript.

Typically, therapeutic success is assessed by the decrease in the extent of the primary and any metastatic diseases lesions. For solid tumors, decreasing tumor size is the primary indicia of successful treatment. Neighboring tissues should be biopsied to determine the extent to which metastasis has occurred. Tissue biopsy methods are known to those skilled in the art. For non-solid tumors, i.e. the leukemias, treatment is monitored primarily by histological examination of the bone marrow for surviving leukemic cells. How-

ev r, a significant number of leukemic cells may still exist when marrow examination provides normal results. For this reason, more recent methods for detecting leukemic cells have focused on detecting the presence of the gene for the relevant oncogene, or its corresponding mRNA, in cells of the bone marrow as a more sensitive test. See for example the following U.S. Patents: 4,681,840, 4,857,466 and 4,874,853. The presence of even a few copies of the target oncogene can be effectively detected by amplification using reverse transcriptase polymerase chain reaction technology. For a detailed discussion of such methods, see for example, Cancer: Principles & Practice of Oncology, edited by V. T. DeVita, S. Hellman and S.A. Rosenberg, J.B. Lippincott Company, Philadelphia, PA (3rd ed., 1989), incorporated herein by reference. Methods for diagnosing and monitoring the progress of neoplastic disorders vary depending upon the nature of the particular disease.

According to one embodiment, B-myb antisense inhibition of cell proliferation is demonstrated below using Balb/c3T3 fibroblasts. These cells have a well-defined growth-factor requirement, which allows determination of the stage in $G_0$/transition that requires B-myb function. B-myb is expressed in Balb/c3T3 fibroblasts at the $G_1$/S boundary with a kinetics similar to that of c-myb in phytohemagglutinin-stimulated normal T-lymphocytes; B-myb is believed to be growth-regulated at the $G_1$/S boundary (Golay et al., Blood 77, 149 (1991); Reiss et al., J. Cell. Physiol. 148, 338 (1991)).

In connection with demonstrating B-myb antisense inhibition of Balb/c3T3 fibroblasts, the oncogenic potential of B-myb was determined by examining the effects of B-myb constitutive expression on the growth of Balb/c3T3. This was achieved by engineering a cell line (Balb/B-myb) which expresses high levels of ex -

genous human B-myb. The cell line was established by transfecting Balb/c3T3 cells with a plasmid containing full-length B-myb cDNA.

To demonstrate that B-myb expression alters the growth characteristics of the transfected cells, Balb/B-myb and control Balb/c3T3 cells transfected with the pSV40 vector lacking the B-myb cDNA in medium containing 1% serum which induces entry into a quiescent state. After 9 days of culture, the number of Balb/B-myb cells was significantly higher than that of the control cells (about $10^4$ cells/well in plates for Balb/B-myb as compared to about x $10^4$ cells of Balb/c3T3 transfected with the vector alone), indicating that Balb/B-myb cells can still proliferate in low serum conditions, although at a reduced rate (doubling time of 48 hours in 1% serum as compared to 24 hours in 10% serum). The reduced serum requirements of randomly picked single clones from different transfections correlated well with mRNA levels of exogenous B-myb, as indicated by the capacity of clones with high B-myb expression to grow in 1% serum, whereas Balb/c3T3 cells require platelet-derived growth and insulin or insulin-like growth factor for survival in serum-free medium. Balb/B-myb cells not only survived but also grew in serum-free medium, although with a very slow (about 96 hours) doubling time. To determine whether the growth-factor independence of Balb/B-myb cells was an indication of progression toward a transformed phenotype, we plated in soft agar both Balb/B-myb and the parental Balb/c3T3 cell line. After 10 days of culture, Balb/B-myb cells formed colonies in agar with high efficiency (about 50%), while Balb/c3T3 cells formed small colonies, with an efficiency of only about 1-5%.

These findings indicate that cells constitutively xpressing B-myb have at least tw charac-

teristics of a tumorigenic cell. First, the cells proliferate in low serum conditions, that is, they are growth factor-independent. Second, they grow in soft agar. Their growth is not contact inhibited.

The effect on cell proliferation of inhibiting B-myb expression with antisense oligonucleotide is demonstrated by Examples 1, 2 and 3. According to Example 1, the blockage of B-myb mRNA function in Balb/c3T3 cells transfected with a human B-myb antisense construct inhibited the proliferation of the Balb/c3T3 cells. According to Example 2, the proliferation of neuroblastoma cells is likewise inhibited by transfection with the antisense construct. In Example 3, the proliferation of neuroblastoma cells was inhibited by exogenous B-myb antisense oligonucleotide.

## Example 1

### Inhibition of Cloning Efficiency of Balb/c3T3 Cells Transfected with a pSV/anti-B-myb Construct

A. pSV/B-myb Construct

A human lymphoma cDNA library cloned in a γgt11 vector was screened with a 1.4 kilobase radiolabelled B-myb fragment (Nomura et al., Nucleic Acids Res. 16, 11075 (1988)). A 1,469-bp fragment was subcloned into an SK-plasmid vector (Stragene, La Jolla, CA). The remaining 5′ portion of the cDNA was cloned by polymerase chain reaction amplification of reverse-transcribed B-myb mRNA from HL-60 cells. Full-length B-myb cDNA was subsequently eluted from the SK vector (pSKB-myb), digested with Cla1 and Xba1 and subcloned into the pSV40 polylinker vector, which contains a polycloning site located in betw en the pSV40 early promoter

and the SV40 polyadenylation signals. The resulting construct was designated pSV/B-myb.

B. pSV/anti-B-myb Construct

The SKB-myb plasmid was linearized by XhoI digestion. The restriction site was filled by Klenow treatment before digesting with SpeI. A 2 kb B-myb cDNA fragment (XhoI blunted-SpeI) was cloned in the antisense orientation with respect to the SV40 promoter into the pSV40-polylinker vector linearized with XbaI, Klenow-filled and subsequently digested with SpeI. The resulting construct was designated pSV/anti-B-myb.

C. Transfection of Balb/c3T3 Cells

Plasmids of the pSV/B-myb and pSV/anti-B-myb constructs (10 $\mu g/10^6$ cells) were transfected into Balb/c3T3 cells by calcium phosphate precipitation in the presence of 1 $\mu g$ of pLHL4 which carries the hygromycin resistance gene (Furukawa et al., Science 250, 805 (1990); Lee et al., Nature 336, 738 (1988); Draetta et al., Oncogene 2, 55 (1988)). Hygromycin resistant clones were selected in hygromycin-containing medium (250 $\mu g/ml$), scored 12 days after transfection and stained with crystal violet. The results are shown in Figures 1A-1C: 1A, pSV40 polylinker-transfected cells; 1B, pSV/B-myb-transfected cells; 1C, pSV/anti-B-myb-transfected cells. A drastically reduced number of hygromycin-resistant colonies (about 90% inhibition) is observed for cells treated with the antisense construct (Fig. 1C), as compared to Balb/c3T3 cells transfected with the vector only (Fig. 1A) or sense construct (Fig. 1B).

**Example 2**

Inhibition of Cloning Efficiency of Neuroblastoma Cells Transfected with a pSV/anti-B-myb Construct

Neuroblastoma cell line LAN-5 (Seeger et al., J. Immunol. 128, 983-989 (1982)) was grown in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO.) supplemented with fetal bovine serum (FEBS) (Sigma). The procedure of Example 1 was repeated substituting LAN-5 cells for Balb/c3T3. Again, a drastically reduced number of hygromycin-resistant colonies was observed for cells treated with the antisense construct (Fig. 2B), as compared to cells transfected with the sense construct (Fig. 2A).

**Example 3**

B-myb Antisense Oligonucleotide Inhibition of Neuroblastoma Cell Proliferation

In a typical experiment, 1 x $10^4$ LAN-5 cells were seeded in the presence of antisense (SEQ ID NO: 5) or sense (SEQ ID NO: 18) phosphorothioate oligodeoxynucleotides (80 μg/ml at zero hours, 40 μg/ml after 18 hours, and 40 μg/ml after 36 hours). Cells were counted after 7 or 9 days. The results are set forth in Fig. 2: C, no oligomer: S, sense oligomer; and AS, antisense oligomer. Cell proliferation was inhibited by the antisense oligomer.

**Example 4**

Bone Marrow Purging with B-myb Antisense Oligonucleotide

Bone marrow is harvested from the iliac bones of a donor und r general anesthesia in an operating room

using standard techniques. Multiple aspirations are taken into heparinized syringes. Sufficient marrow is withdrawn so that the marrow recipient will be able to receive about 4 x $10^8$ to about 8 x $10^8$ processed marrow cells per kg of body weight. Thus, about 750 to 1000 ml of marrow is withdrawn. The aspirated marrow is transferred immediately into a transport medium (TC-199, Gibco, Grand Island, New York) containing 10,000 units of preservative-free heparin per 100 ml of medium. The aspirated marrow is filtered through three progressively finer meshes until a single cell suspension results, i.e., a suspension devoid of cellular aggregates, debris and bone particles. The filtered marrow is then processed further into an automated cell separator (e.g., Cobe 2991 Cell Processor) which prepares a "buffy coat" product, (i.e., leukocytes devoid of red cells and platelets). The buffy coat preparation is then placed in a transfer pack for further processing and storage. It may be stored until purging in liquid nitrogen using standard procedures. Alternatively, purging can be carried out immediately, then the purged marrow may be stored frozen in liquid nitrogen until it is ready for transplantation.

The purging procedure may be carried out as follows. Cells in the buffy coat preparation are adjusted to a cell concentration of about 2 x $10^7$/ml in TC-199 containing about 20% autologous plasma. B-myb antisense oligodeoxynucleotide, for example, in a concentration of about 50-100 μg/ml, is added to the transfer packs containing the cell suspension. The transfer packs are then placed in a 37°C waterbath and incubated for 18 - 24 hours with gentle shaking. The cells may then either be frozen in liquid nitrogen or washed once at 4°C in TC-199 containing about 20% autolog us plasma to r move unincorp rated olig mer. Washed cells are

then infused into the recipient. Care must be taken to work under sterile conditions wherever possible and to maintain scrupulous aseptic techniques at all times.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

All references cited herein with respect to synthetic, preparative and analytical procedures are incorporated by reference.

## SEQUENCE LISTING

(1) **GENERAL INFORMATION:**

(i) **APPLICANT:** Thomas Jefferson University

(a) **INVENTOR:** Calabretta, Bruno

(ii) **TITLE OF INVENTION:** Antisense Oligonucleotides to B-myb Proto-oncogene.

(iii) **NUMBER OF SEQUENCES:** 18

(iv) **CORRESPONDENCE ADDRESS:**

(A) **ADDRESSEES:** Thomas Jefferson University

(B) **STREET:** 10th and Locust Streets

(C) **CITY:** Philadelphia

(D) **STATE:** Pennsylvania

(E) **COUNTRY:** U.S.A.

(F) **ZIP:** 19107

(v) **COMPUTER READABLE FORM:**

(A) **MEDIUM TYPE:** Diskette, 3.50 inch, 720 Kb

(B) **COMPUTER:** IBM PS/2

(C) **OPERATING SYSTEM:** MS-DOS

(D) **SOFTWARE:** WordPerfect 5.1

(vi) **CURRENT APPLICATION DATA:**

(A) **APPLICATION NUMBER:**

(B) **FILING DATE:**

(C) **CLASSIFICATION:**

(vii) **PRIOR APPLICATION DATA:**

(A) **APPLICATION NUMBER:** 07/956,698

(B) **FILING DATE:** 01 October 1992

(viii) **ATTORNEY/AGENT INFORMATION:**

(A) **NAME:** Monaco, Daniel A.

(B) **REGISTRATION NUMBER:** 30,480

(C) **REFERENCE/DOCKET NUMBER:** 8321-3

(ix) **TELECOMMUNICATION INFORMATION:**

(A) **TELEPHONE:** (215) 568-8383

(B) **TELEFAX:** (215) 568-5549

(C) **TELEX:** None

(2) **INFORMATION FOR SEQ ID NO:1:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 50 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:1:**

TCCTGGTAGT GCAGCTCATC CAGATCCTCG 30

CAGCGCGTCC GCCGAGACAT 50

(2) **INFORMATION FOR SEQ ID NO:2:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 30 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:2:**

CAGATCCTCG CAGCGCGTCC GCCGAGACAT 30

(2) **INFORMATION FOR SEQ ID NO:3:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 29 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:3:**

AGATCCTCGC AGCGCGTCCG CCGAGACAT 29

(2) **INFORMATION FOR SEQ ID NO:4:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 28 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:4:**

GATCCTCGCA GCGCGTCCGC CGAGACAT 28

(2) **INFORMATION FOR SEQ ID NO:5:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 27 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:5:**

ATCCTCGCAG CGCGTCCGCC GAGACAT 27

(2) **INFORMATION FOR SEQ ID NO:6:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 26 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:6:**

TCCTCGCAGC GCGTCCGCCG AGACAT 26

(2) **INFORMATION FOR SEQ ID NO:7:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 25 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:7:**

CCTCGCAGCG CGTCCGCCGA GACAT 25

(2) **INFORMATION FOR SEQ ID NO:8:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 24 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:8:**

CTCGCAGCGC GTCCGCCGAG ACAT 24

(2) **INFORMATION FOR SEQ ID NO:9:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 23 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:9:**

TCGCAGCGCG TCCGCCGAGA CAT 23

(2) **INFORMATION FOR SEQ ID NO:10:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 22 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:10:**

CGCAGCGCGT CCGCCGAGAC AT 22

(2) **INFORMATION FOR SEQ ID NO:11:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 21 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:11:**

GCAGCGCGTC CGCCGAGACA T 21

(2) **INFORMATION FOR SEQ ID NO:12:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 20 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:12:**

CAGCGCGTCC GCCGAGACAT 20

(2) **INFORMATION FOR SEQ ID NO:13:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 19 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:13:**

AGCGCGTCCG CCGAGACAT 19

(2) **INFORMATION FOR SEQ ID NO:14:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 18 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:14:**

GCGCGTCCGC CGAGACAT 18

(2) **INFORMATION FOR SEQ ID NO:15:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 17 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:15:**

CGCGTCCGCC GAGACAT 17

(2) **INFORMATION FOR SEQ ID NO:16**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 16 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:16:**

GCGTCCGCCG AGACAT 16

(2) **INFORMATION FOR SEQ ID NO:17:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 15 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:17:**

CGTCCGCCGA GACAT 15

(2) **INFORMATION FOR SEQ ID NO:18:**

(i) **SEQUENCE CHARACTERISTICS:**

(A) **LENGTH:** 25 Nucleotides

(B) **TYPE:** nucleic acid

(C) **STRANDEDNESS:** single stranded

(D) **TOPOLOGY:** linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:18:**

ATGTCGGC GGACGCGCTG CGAGGAT 25

## CLAIMS

1. A method for the treatment of a neoplastic disease characterized by the activation of B-myb expression comprising administering to an individual in need of such treatment an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the B-myb gene, said oligonucleotide being hybridizable to said mRNA transcript.

2. A method according to claim 1 wherein the oligonucleotide is an at least 8-mer.

3. A method according to claim 2 wherein the oligonucleotide does not hybridize to c-myb mRNA.

4. A method according to claim 2 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

5. A method according to claim 2 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb mRNA lying within about 50 nucleotides of the translation initiation codon.

6. A method according to claim 5 wherein the oligonucleotide is a phosphorothioate oligodeoxynucleotide or methylphosphonate oligodeoxynucleoside.

7. A method according to claim 2 wherein the oligonucleotide is an oligodeoxynucleotide having a deoxynucleotide sequence complementary to a portion

of the B-myb mRNA transcript including the translation initiation codon of said transcript.

8. A method according to claim 2 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

9. A method according to claim 8 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or a phosphorothioate oligonucleotide.

10. A method according to claim 8 wherein the oligonucleotide is from a 15-mer to 30-mer.

11. A method according to claim 10 wherein the oligonucleotide is from a 18-mer to 26-mer.

12. A method according to claim 11 wherein the oligonucleotide is from a 18-mer to 21-mer.

13. A method according to claim 5 wherein the oligonucleotide is an oligodeoxynucleotide having a nucleotide sequence of SEQ ID NO:1, or an at least 12-mer portion thereof.

14. A method according to claim 1 wherein the neoplastic disease is selected from the group consisting of malignant melanoma, neuroectodermal cancers, breast cancer, prostate carcinoma, colon cancer, renal carcinoma, leukemia and lymphoma.

15. A method according to claim 1 wherein the antisense oligonucleotide is administered locally.

16. A method for purging bone marrow of neoplastic cells comprising

treating bone marrow cells aspirated from an individual afflicted with a neoplastic disease characterized by the activation of B-myb expression with an effective amount of an oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the B-myb gene, said oligonucleotide being hybridizable to said mRNA transcript, and

returning the thus-treated cells to the body of the afflicted individual.

17. A method according to claim 16 wherein the oligonucleotide is an at least 8-mer.

18. A method according to claim 17 wherein the oligonucleotide is a alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

19. A method according to claim 17 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb mRNA lying within about 50 nucleotides of the translation initiation codon.

20. A method according to claim 17 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

21. An oligonucleotide comprising from 8 to 50 nucleotides which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the B-myb gene, said oligonucl otide b ing hybridizable to said mRNA transcript.

22. An oligonucleotide according to claim 21 which does not hybridize to c-myb mRNA.

23. An oligonucleotide according to claim 21 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

24. An oligonucleotide according to claim 21 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb transcript lying within about 50 nucleotides of the translation initiation codon.

25. An oligonucleotide according to claim 21 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb mRNA transcript including the translation initiation codon of said transcript.

26. An oligonucleotide according to claim 21 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

27. An oligonucleotide according to claim 26 wherein the oligonucleotide is from a 15-mer to a 30-mer.

28. An oligonucleotide according to claim 27 wherein the oligonucleotide is from a 18-mer to a 26-mer.

29. An oligonucleotide according to claim 28 wherein the oligonucleotide is from a 18-mer to a 21-mer.

30. An oligonucl otide according to claim 21 wherein the oligonucleotide is an oligodeoxynucleotide having a nucleotide sequence corresponding to SEQ ID NO:1, or at least 12-mer portion thereof.

31. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and at least one oligonucleotide which has a nucleotide sequence complementary to at least a portion of the mRNA transcript of the B-myb gene, said oligonucleotide being hybridizable to said mRNA transcript.

32. A composition according to claim 31 wherein the oligonucleotide does not hybridize to c-myb mRNA.

33. A composition according to claim 31 wherein the oligonucleotide is an alkylphosphonate oligonucleoside or phosphorothioate oligonucleotide.

34. A composition according to claim 31 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb mRNA transcript lying within about 50 nucleotides of the translation initiation codon.

35. A composition according to claim 31 wherein the oligonucleotide has a nucleotide sequence complementary to a portion of the B-myb mRNA transcript including the translation initiation codon of said transcript.

36. A composition according to claim 31 wherein the oligonucleotide comprises from a 12-mer to a 40-mer oligodeoxynucleotide.

37. A composition according to claim 36 wherein the oligonucleotide is from a 15-mer to a 30-mer.

38. A composition according to claim 37 wherein the oligonucleotide is from a 18-mer to 26-mer.

39. A composition according to claim 38 wherein the oligonucleotide is from a 18-mer to 21-mer.

40. A composition according to claim 31 wherein the oligonucleotide is an oligodeoxynucleotide having a nucleotide sequence corresponding to SEQ ID NO:1, or at least a 12-mer portion thereof.

41. An artificially-constructed gene comprising a transcriptional promotor segment and a segment containing a B-myb DNA in inverted orientation such that transcription of said artificially-constructed gene produces RNA complementary to at least a portion of the mRNA transcript of the B-myb gene.

42. A method according to claim 41 wherein the promotor segment comprises SV40 promotor.

43. A method for inhibiting the proliferation of neoplastic cells characterized by the activation of B-myb expression comprising introducing into such cells an artificially-constructed gene which, upon transcription in said cells, produces RNA complementary to the mRNA transcript of the B-myb gene.

44. A method according to claim 43 wherein the artificially-constructed gene is introduced into said cells by transfection, by a transducing viral vector or by microinjection.

FIG. 1A

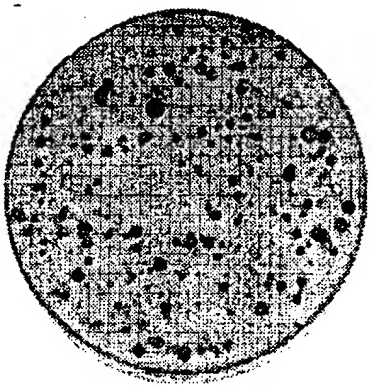


FIG. 1B

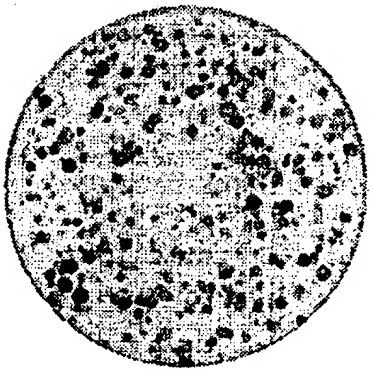


FIG. 1C

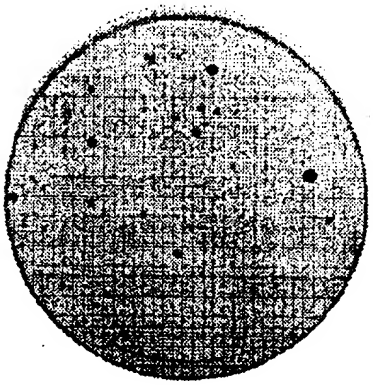


FIG. 2A

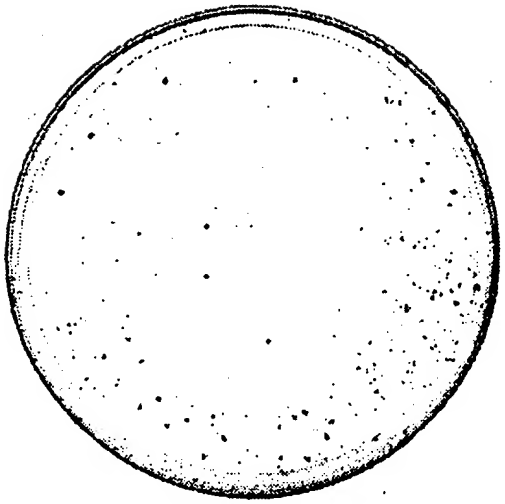
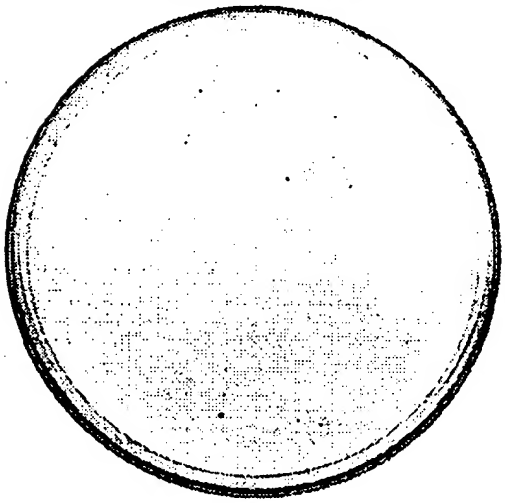


FIG. 2B



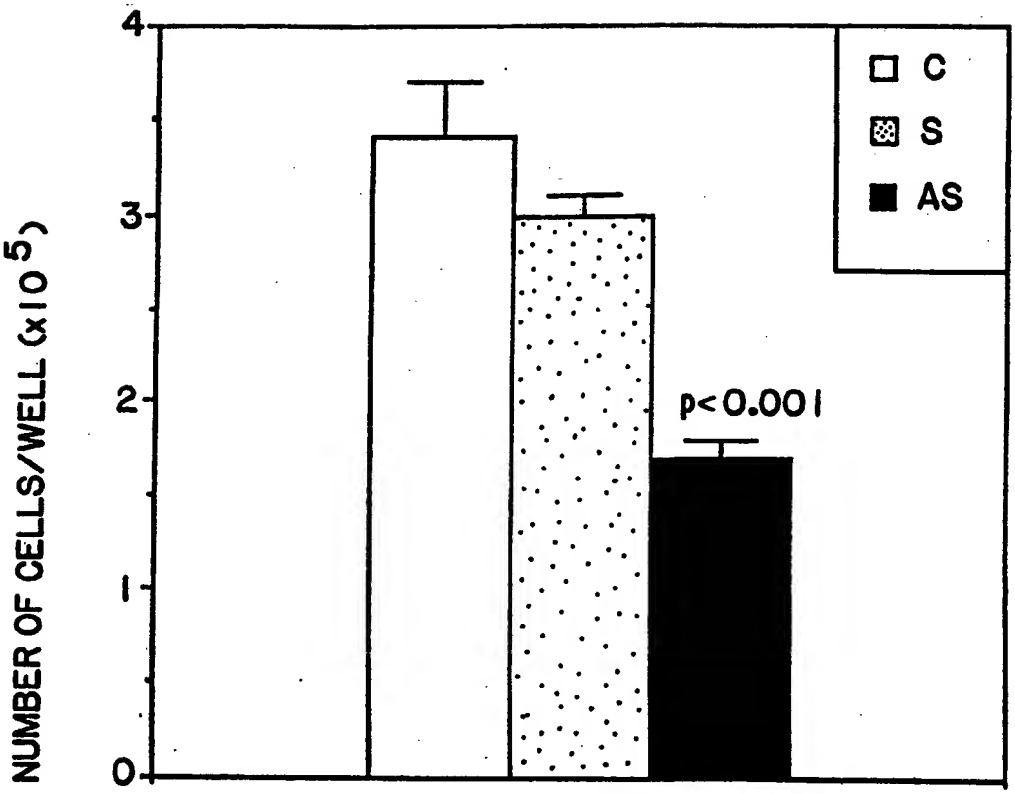



FIG. 3

# INTERNATIONAL SEARCH REPORT

[redacted]ional application No.
[redacted]/US93/09013

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :A61K 48/00; C07H 21/04; C12N 5/10, 15/70
US CL :514/44; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 29, 320.1; 514/44; 536/24.5; 935/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, BIOSIS, MEDLINE, GENBANK, GENESEQ, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BLOOD, Volume 79, Number 10, issued 15 May 1992, M. Arsura et al., "B-myb Antisense Oligonucleotides Inhibit Proliferation of Human Hematopoietic Cell Lines", pages 2708-2716, see page 2710. | 21, 22, 24-30 |
| X | NATURE, Volume 359, issued 03 September 1992, M. Simons et al., "Antisense c-myb Oligonucleotide Inhibit Intimal Arterial Smooth Muscle Cell Accumulation in vivo", pages 67-70, see page 68. | 31, 33, 34, 36-40 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be part of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

| | |
|---|---|
| Date of the actual completion of the international search<br>13 December 1993 | Date of mailing of the international search report<br>30 DEC 1993 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE | Authorized officer<br>PAUL B. TRAN, PH.D.<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09013

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 266, Number 27, issued 25 September 1991, M-Y Chiang et al., "Antisense Oligonucleotides Inhibit Intercellular Adhesion Molecule 1 Expression by Two Distinct Mechanisms", pages 18162-18171, see pages 18163-18166. | 1-40 |
| Y | NUCLEIC ACIDS RESEARCH, Volume 16, Number 23, issued 1988, N. Nomura et al., "Isolation of Human cDNA Clones of myb-related Genes, A-myb and B-myb", pages 11075-11089, see page 11080. | 1-40 |
| Y | BLOOD, Volume 77, Number 1, issued 01 January 1991, J. Golay et al., "Expression of c-myb and B-myb, but not A-myb, Correlates With Proliferation in Human Hematopoietic Cells", pages 149-158, see Abstract. | 1-40 |
| Y | PROC. NATL. ACAD. SCI., Volume 88, issued September 1991, S. Agrawal et al., "Pharmacokinetics, Biodistribution, and Stability if Oligodeoxynucleotide Phosphorothioates in Mice", pages 7595-7599, see pages 7595-7596. | 4, 6, 9, 18, 23, 33 |
| Y | US, A, 5,087,617 (SMITH) 11 FEBRUARY 1992, see entire document. | 16-20 |
| Y | US, A, 5,098,890 (GEWIRTZ ET AL.) 24 MARCH 1992, see entire document. | 1-40 |

INTERNATIONAL SEARCH REPORT

[redacted]ional application No.
[redacted]CT/US93/09013

**Box I Observations where certain claims were found unsearchable (C ntinuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:
(Telephone Practice)
Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-40

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment f additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-40, drawn to B-myb antisense oligonucleotides, pharmaceutical compositions thereof (16-40) and methods to treat neoplastic disease and purging bone marrow using thereof (1-15), classified in Class 514, subclass 44 and Class 536, subclass 24.5.

II. Claims 41-44, drawn to B-myb antisense RNA construct and methods of use thereof, classified in Class 435, subclasses 29 and 320.1.

Unity of invention is lacking for the following reasons:

Inventions I and II are independent and distinct because they are drawn to distinct products and their respective uses. Invention I is drawn to an oligonucleotide whereas Invention II is to an RNA- expressing vector. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Note that PCT Rule 13 does not provide for multiple products within a single application.